

Characterizing the Dynamics of Fluorescent Molecule Delivery Using Reversible Membrane Permeabilization with Streptolyosin-O

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Characterizing the Dynamics of Fluorescent Molecule Delivery Using Reversible Membrane Permeabilization with Streptolyosin-O

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SUMMARY

The purpose of this study is to characterize the delivery dynamics of single-molecule fluorescent probes, MTRIPS (multiply labeled tetravalent RNA imaging probes), with Streptolysin-O (SLO) reversible membrane permeabilization. The delivery of MTRIPs was quantified in A549 human adenocarcinoma lung cells. Increase in MTRIP delivery concentrations positively correlated to high MTRIP concentrations in the cytoplasm of the cells. Conversely, the use of varying SLO concentrations with a fixed MTRIP concentration exhibited no distinct change in intracellular MTRIP concentrations, except for high SLO concentrations specifically at 50 U mL^{-1} . Additionally, the effects of MTRIP delivery with SLO were observed in MDCK, Vero, U2OS, and HEp2 cells. Observable differences in intracellular MTRIP concentrations were observed in MDCK, U2OS, and HEp2 cells. However, even at high SLO concentrations, the intracellular MTRIP concentration was not significant in Vero cells. This understanding of the dynamics of SLO delivery for fluorescent probes is crucial to targeting endogenous RNA and proteins to improve *in vitro* imaging techniques.

INTRODUCTION

Almost all cellular function is regulated by complex interactions of macromolecules, such as proteins, lipids, DNA, and RNA. These molecular interactions are the basis of reproduction, metabolism, maintenance, growth, and, essentially, life. Disruption in any of these molecular interactions could lead to the onset or progression of disease. Therefore, identification of these specific interactions and their components is vital to understanding the fundamental mechanisms of cellular processes in order to deter the proliferation of disease and find a method for treatment.

Fluorescence microscopy is a vital tool for studying these molecular interactions within cells. It has been useful for studying cells *in vivo*, within a living organism, and *in vitro*, within a controlled environment. Most fluorescence microscopy studies involve dual-color images of specific molecules that demonstrate colocalization, or the degree of which two signals overlap when collected in separate detection channels for specific molecules [1]. The high degree of colocalization of certain molecules indicates close proximity and the likelihood of nonrandom interactions [1]. Colocalization allows for the visualization of these events but requires single-molecule sensitivity in order to track the progression of specific, individual molecules.

To track low abundance molecules, such as RNA or proteins, single molecule-sensitive fluorescent probes are used and delivered into cells. Single molecule-sensitive fluorescent probes have been used to detect conformational changes, protein expression, localization, trafficking and diffusion *in vitro* [2]. However, to track individual molecules within a cell, the single molecule-sensitive probes must be introduced into the

intracellular environment. In order to introduce these single molecule-sensitive probes into the cell, the plasma membrane must be bypassed. The plasma membrane forms a selectively permeable barrier that separates the cell from the exterior environment and regulates small molecule transport in and out of the cell [3]. To facilitate the visualization of RNA or proteins within the cell, exogenous fluorescent probes that bind to specific molecules within the cell are delivered across the plasma membrane. To avoid degradation problems related to the endocytic pathway, where exogenous molecules are brought into the cell by vesicles, non-endocytic methods have been developed. Microinjection, electroporation, cell-penetrating peptides, and reversible membrane permeabilization are known methods for delivering probes into the cell by bypassing the plasma membrane barrier. With the exception of cell membrane permeabilization, these methods are technically demanding and restricted to small cell numbers [4].

Microinjection and electroporation are two well-established methods commonly used to introduce fluorescent probes into the cell. The microinjection procedure requires direct visualization of the cell and the use of a fine-tipped glass microcapillary to inject [5]. However, it is invasive, may interfere with metabolic processes and mRNA expression, and only allows for delivery of very small sample volumes into the cell [6]. The increase in intracellular volume may even cause mechanical damage to the cell. Additionally, this procedure is technically demanding, time consuming, cost ineffective, and can only be performed in a small number of cells [6]. Electroporation is a technique that utilizes an electric pulse to bypass the plasma membrane for fluorescent probes to enter. This technique has been found to be less efficient in exogenous probe delivery and has a lower rate of cell viability than other methods [5, 7].

Although microinjection and electroporation are practiced methods for probe delivery, each method has limitations that affect the efficiency of delivery. In a study performed by Delehanty et al., microinjection and electroporation are used to deliver quantum dots, or luminescent semiconductor nanocrystals, to an intracellular environment. Both methods bypass the endocytic pathway and deliver materials without the need for endosomal escape. However, quantum dot delivery varies between the two methods. Electroporation was found to be better technique for delivering large quantities of quantum dots ($\sim 10^6$). Although electroporation is successful in delivering larger quantities, it was noted that there is usually a high cellular mortality rate due to the subjugation of membrane permeabilization and a delivery of a strong electrical pulse. Additionally, there were problems with intracellular aggregation of the electroporated particles. In comparison, microinjection induced a lower rate of cell death; however, only a small number of cells were successfully injected. This is due to individual cell selection, the need for a well-trained operator, and physical constraints such as cell morphology, membrane thickness, and cell height. Microinjection is also costly, with devices ranging from \$30-\$40,000, relative to electroporation devices which are less than \$5,000 [5].

An alternative method of delivery is with the use of cell-penetrating peptides to deliver fluorescent probes by translocating them through the plasma membrane. This process involves binding the molecule of interest to a cell-penetrating peptide in order for it to translocate into the cell. This technique has been proven to be useful for the delivery of fluorescent probes; however, cell-penetrating peptides have shown to be cargo-specific in their mechanisms of action and to have toxic effects on the cell at high concentrations.

Furthermore, since the mechanism for this process is not well understood, this method of delivery could potentially have additional unknown side effects [8].

Although cell-penetrating peptide delivery may be a valuable, alternative way to deliver probes, there have been multiple limitations observed with this delivery method. In a study performed by Zorko et al., cell-penetrating peptides were determined to be useful for drug delivery of small molecules, proteins, and supramolecular particles. In this study, the internalization of cell-penetrating peptides and their cargo were monitored, and it was determined that the internalization of cell-penetrating peptides occurred at a fast rate (< 1 hour of incubation). Small cargoes were determined to have no effect on the rate of internalization, while larger hydrophilic cargos (proteins) were internalized more slowly. Additionally, more than 50% of the maximal internalization of cell-penetrating peptides was achieved. Even though the results were promising for drug delivery, this relatively new process is still not well understood. The mechanism of internalization has not yet been resolved; however, it was shown that some cell-penetrating peptides rely on the cellular membrane composition or may be linked to endocytic pathways, which requires additional mechanisms for endosomal escape. Cell-penetrating peptides have also shown some levels of toxicity on membranes of cells and organelles, which result in specific interactions of cell-penetrating peptides with wall components. Some specific cell-penetrating peptides have even been observed to inhibit GTPase activity within the cell. The stability of cell-penetrating peptides has also been problematic. High extracellular stability could aid in efficient cargo delivery; however, high intracellular stability could result in the accumulation of cell-penetrating peptides that could engender side effects [8].

In contrast to these three costly, technically demanding, and ambiguous methods, Steptolysin-O (SLO) reversible membrane permeabilization is a simple, effective technique for probe delivery that maintains cell viability, does not utilize endosomes, and prevents degradation of probes [7]. Streptolysin-O is a toxin produced by most strains of beta- hemolytic group A Streptococci and is used to perform reversible membrane permeabilization, a process in which SLO proteins bind to surface cholesterol on cell membranes and form functional pores [9] that can reach up to 35 nanometers in diameter [4]. The formation of these pores can be reversed in the presence of high magnesium and calcium environments, allowing for reversible membrane permeabilization. The use of SLO enhances the intracellular uptake of large molecules within cells [7] and is useful in the delivery of nanoparticles into living cells, including exogenous fluorescent probes [10].

The efficacy of fluorescent molecule SLO delivery was shown to achieve higher intracellular concentrations [6] without significantly effecting cellular functions[4]. In a study performed by Spiller et al, the delivery of oligonucleotides utilizing electroporation and SLO reversible membrane permeabilization were compared. It was shown that most cells permeabilized with SLO contained higher concentrations of oligonucleotides than electroporated cells. Additionally, cells treated with a supraoptimal dose of SLO, designed to have equivalent levels of toxicity to electroporation (12-14% cell death), had a rate of less than 5% cell death.[7] Furthermore in a corresponding study performed by Walev et al, the effect of SLO permeabilization on cellular functions was proven to be minimal. SLO permeabilized cells were shown to remain viable for days, to retain endocytic functions, and to proliferate normally. Initially, cells treated with SLO

exhibited 80% ATP depletion but were able to gradually recuperate 50-90% of their original ATP levels after four hours. It was also shown that cells treated with SLO showed no changes in glycosylation patterns, which indicates that cellular components were not lost to the extracellular environment.[4]

The use of SLO reversible membrane permeabilization appears to be the optimal technique to deliver exogenous probes into a cell due to the lack of endosomal uptake, the high levels of cell viability, and the enhanced intracellular uptake of exogenous molecules. Although the molecular mechanisms for SLO are well known, the dynamics of SLO delivery for fluorescent molecules are not yet well characterized. It is not well understood how the concentration of fluorescent probe delivered to the cells or the concentration of SLO used affects the intracellular concentrations of probe taken up by the cell. Expanding current research with SLO may lead to advances in molecular fluorescent-imaging techniques by optimizing intracellular concentrations of fluorescent molecules within cells. This in turn can lead to a better imaging or tracking of single endogenous RNA or protein molecules.

The dynamics of SLO delivery were observed by delivering fluorescent probes into the cytoplasm of A549 lung carcinoma cells. It was hypothesized that the intracellular concentration of fluorescent probes can be controlled by manipulating the concentrations of probe delivered or by controlling the amount SLO used during the delivery. In addition, the effects of fluorescent probe delivery using SLO varying were observed with varying cell types.

MATERIALS AND METHODS

MTRIPS (multiply labeled tetravalent RNA imaging probes) were delivered into A549 lung carcinoma cells using Streptolysin-O (SLO). Images were captured using fluorescence microscopy and data was analyzed using Volocity software.

MTRIPs. MTRIPs were created by binding 2' O-methyl RNA-DNA chimera nucleic acid ligands with 5' biotin and multiple dT-C6-NH₂ modifications, manufactured by Biosearch Technologies Inc. Streptavidin purchased from Pierce were used for the core of the MTRIPs. Probes were assembled by resuspending the purified RNA-DNA ligands with bound fluorophores in phosphate-buffered saline (PBS; pH 7.4) and mixed at a 10:1 molar ratio with streptavidin for 1 hour at room temperature (18-22°C) as seen in Figure 1. Free ligands were removed with a 30 kDa Nanosep spin column and stored at 1 μM final concentration in PBS at 4°C.

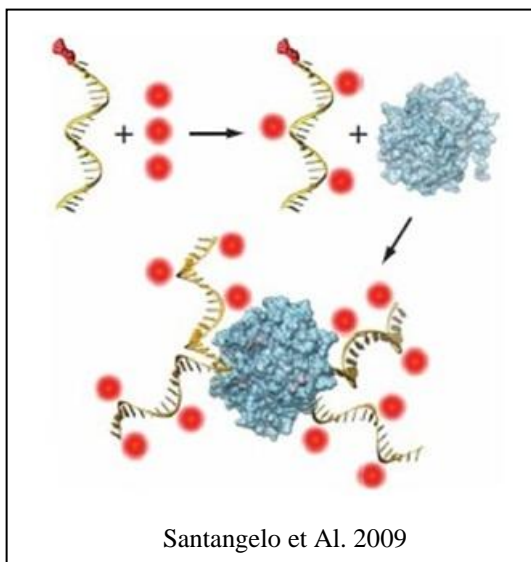


Figure 1. RNA-DNA chimera nucleic acid ligands were bound to Cy3B fluorophores. Streptavidin was used as the core and bound to the ligands with fluorophores, and excess ligand was removed with a spin column.

Cells. A549 lung carcinoma cells (American Type Culture Collection CCL-185) were grown in DMEM (Sigman Aldrich) with 10% fetal bovine serum (FBS; Hyclone) with 100 U^{-1} of penicillin and 100 mg ml^{-1} of streptomycin. Identical cell culture procedures were used for the (MDCK) Madin-Darby Canine Kidney epithelial cells, (Vero C1008) African green monkey kidney epithelia cells, (U2OS) human osteosarcoma cells, and (HEp2) human epidermoid cancer cells.

Probe Delivery. MTRIPs were delivered into A549 cells with streptolysin O (Sigma S-5265) as seen in Figure 2. SLO at 2 U mL^{-1} was combined with TCEP bond breaker and incubated at 37°C for an hour. After incubation, SLO was diluted 1:10 with Optimem. Then, optimem diluted MTRIPs were added to the SLO delivery solution. Cells used for delivery were grown in complete medium and were initially washed with PBS. They were then incubated with SLO and probe for ten minutes at 37°C . The mixture of SLO and probes was removed and replaced with fresh, complete growth medium for fifteen minutes at 37°C .

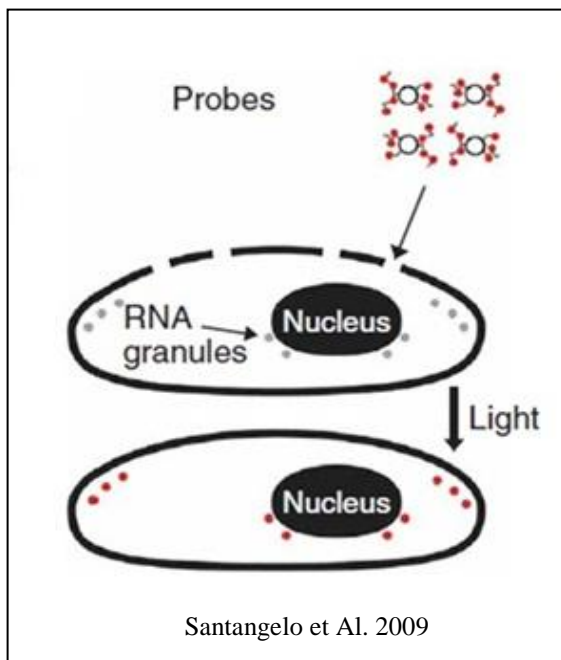


Figure 2. MTRIPs were delivered into cells using SLO delivery solution. Pores formed were then recovered in a calcium and magnesium rich environment using complete DMEM media with FBS, penicillin, and streptomycin.

Staining. A549 cells were fixed in 4% paraformaldehyde solution for ten minutes at room temperature. They were then washed three times with PBS and permeabilized with 0.2% Triton-X 100 for five minutes at room temperature. After permeabilization, the cells were washed in PBS, blocked for thirty minutes in 5% BSA (ultrapure). After they were washed in PBS, the cells were stained with Phalloidin 488 for thirty minutes at 37°C and washed three times with PBS. Nuclei were stained with DAPI for five minutes at room temperature and then mounted with Prolong antifade reagent on glass slides.

Fluorescence imaging. Immobilized Cy3B probes on the glass surface were imaged with a Zeiss Axiovert 200M microscope with an X63, NA=1.4 Plan-Apochromat objective using chroma 49004 ET-Cy3 and 49006 ET-Cy5 filter sets with 500-ms exposures. An EXFO excite 120 light source with a ND (neutral density) = 0.4% (40% transmission) was used for fluorescence excitation and a Hamamatsu ORCA-ER Ag camera for taking digital images. All images were collected with Volocity software. Seven to ten cell images were taken for each case using a Cy3B channel for probe, a FITC channel for actin filaments and cell structure, and a DAPI channel for the nucleus.

Data Analysis. MTRIPs were quantified using Volocity tracking software. Images were deconvolved, and probes were identified by fluorescent intensity and size. The quantity of probes present inside the cells were counted and analyzed. Probes were counted in deconvolved images using SD intensity. They were then clipped to the region of interest (ROI). Touching objects were separated if the object was greater than $0.05\mu\text{m}^3$, and objects were excluded by size if they were greater than $0.01\mu\text{m}^3$.

RESULTS

In order to determine the rate of MTRIPs present in the cytoplasm of cells, respiratory syncytial virus (RSV) targeted MTRIPs were delivered to noninfected human adenocarcinoma A549 cells. The non-specific binding of RSV MTRIPs allowed for probes to freely move through pores created by SLO and into the cytoplasm. MTRIP concentrations were then visualized using fluorescence microscopy and were counted using Volocity's tracking software. The quantity of probes present in the cell was collected from seven to ten cells for each case.

Initially, probe delivery was observed in the no SLO and SLO delivery cases in Figure 3. Probe was delivered at a 1nM concentration in Optimem control and 2U mL⁻¹ of SLO. In the no SLO case, fewer MTRIPs were observed than in the SLO condition. Some MTRIPs were observed due to residual probe adhering to the surface of cells; however, no probe appeared to be present inside the cytoplasm of cells. Although residual binding of probe to the surface of cells is present, the quantity of probes present inside the cell is significantly greater than the quantity of probes adhered to cell surfaces.

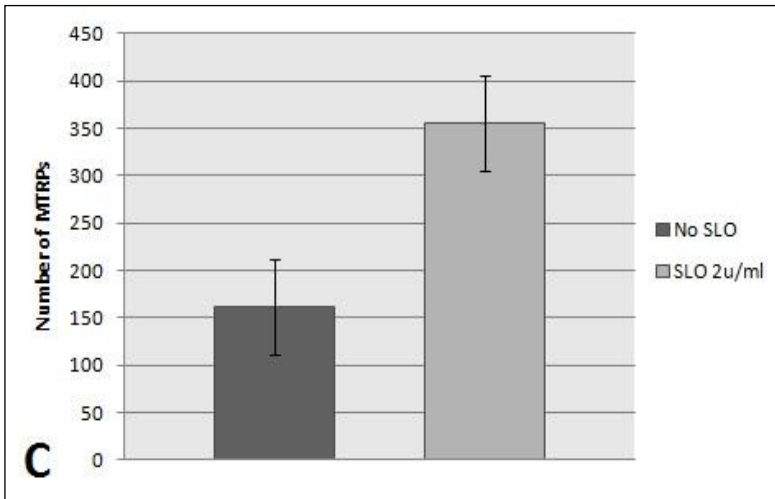
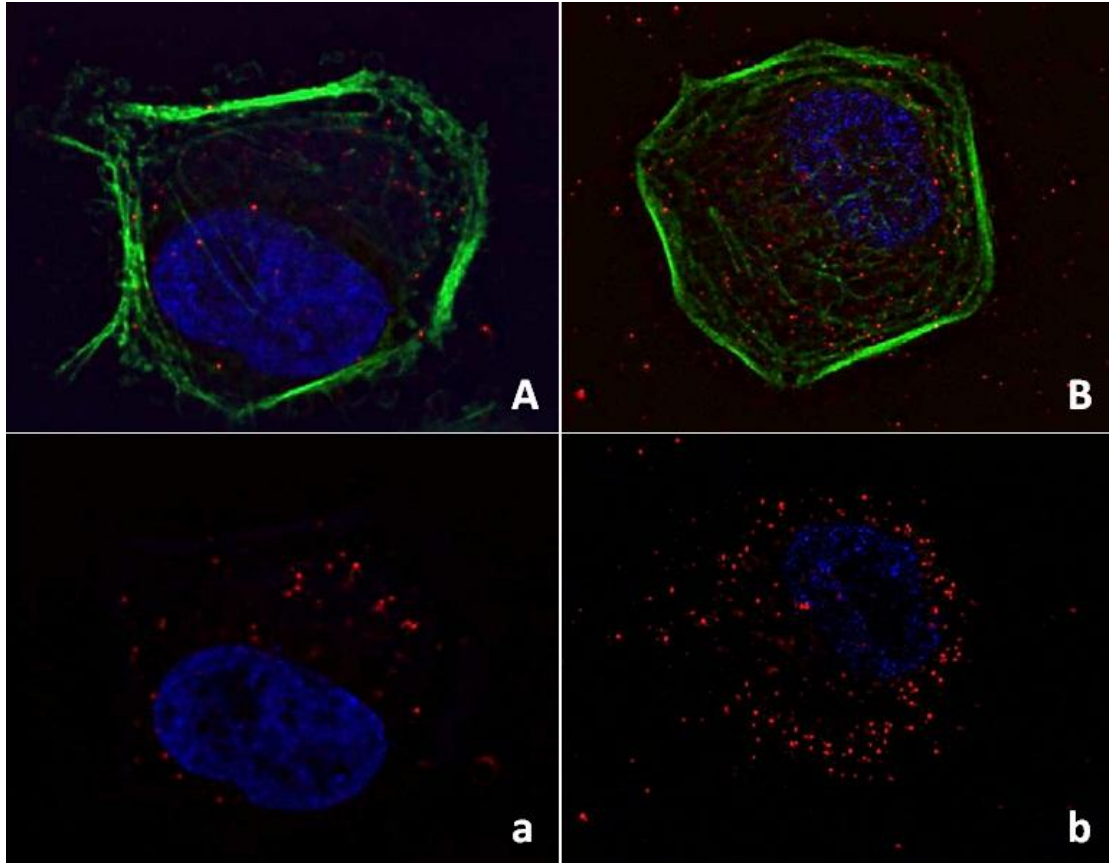


Figure 3. 1nM of RSV Cy3B MTRIPs were delivered in OptiMem for the control case, and 1nM of RSV MTRIP in 2U mL⁻¹ of SLO diluted 1:10 with OptiMem in A549 cells. Cells were cultured in DMEM media with 10% FBS and with 100 U⁻¹ of penicillin and 100 mg mL⁻¹ of streptomycin. Cells were washed with Ca and Mg free PBS before MTRIP delivery with SLO. MTRIPs in OptiMem and SLO were delivered to the cells and incubated for ten minutes at 37°C. Following the delivery, cells were recovered in complete DMEM media for fifteen minutes at 37°C, fixed in 4% paraformaldehyde for ten minutes at room temperature, and stained. Images were captured and probes were quantified with Volocity software. Green represents the cell structure; blue represents the nuclei (DAPI); and red represents the MTRIPs (Cy3): lower case images indicate the same cell but only with the DAPI and Cy3 channels **A**) Single cell with a no SLO MTRIP delivery **B**) Single cell with 2U mL⁻¹ SLO MTRIP delivery **C**) Comparison of average MTRIPs in the cell for the no slo and slo delivery cases

The dependence of MTRIP delivery based on on probe concentration was initially tested as seen in Figure 4. Varying concentrations of MTRIP at 5nM, 10nM, and 30nM were delivered with SLO at 2U mL^{-1} . These results indicated a linear trend in which higher MTRIP delivery concentrations result in higher delivered concentrations into the cytoplasm. A large increase of MTRIPs inside the cell was observed from 5nM to 10nm and from 10nM to 30nM. This result indicated that the more available probe delivered with SLO effectively increased the amount of probe present inside the cell.

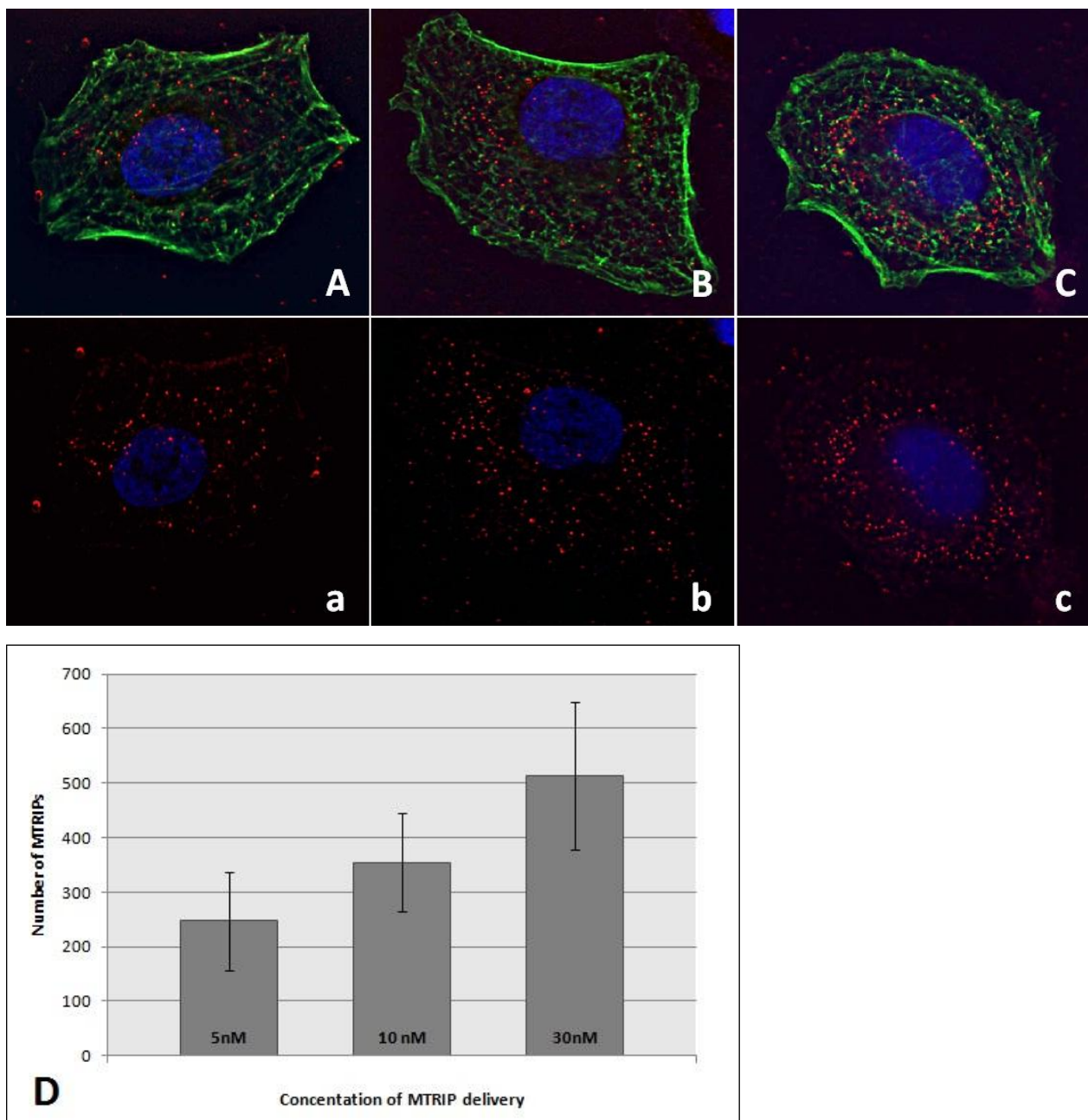
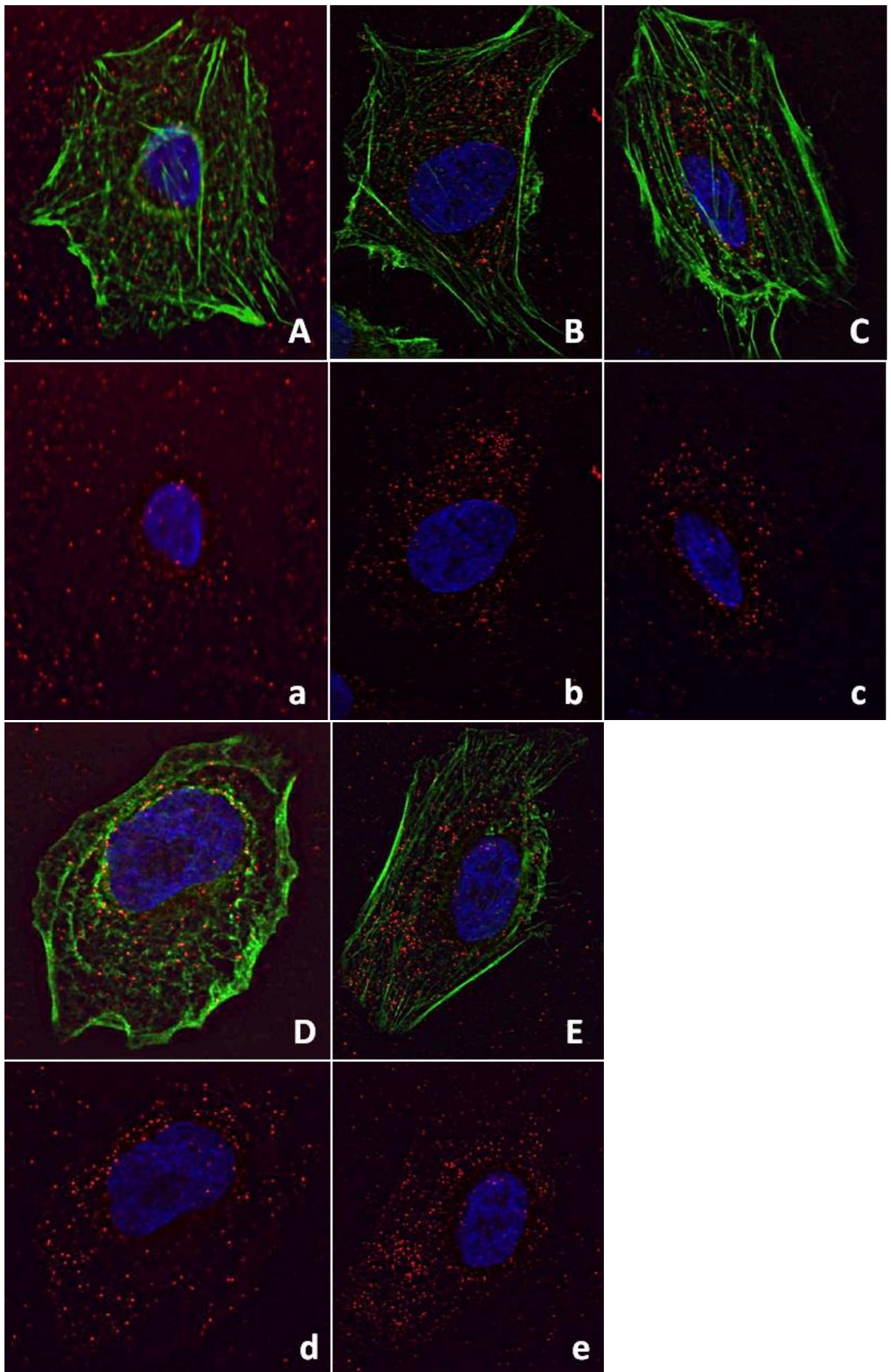


Figure 4. 5nM, 10nM, and 30nM of RSV Cy3B MTRIPs were delivered in 2U mL^{-1} of SLO diluted 1:10 with Optimem in A549 cells. Cells were cultured in DMEM media with 10% FBS and with 100 U^{-1} of penicillin and 100 mg mL^{-1} of streptomycin. Cells were washed with Ca and Mg free PBS before MTRIP delivery with SLO. 5nM, 10nM, and 30nM of RSV Cy3B MTRIPs were delivered in 2U mL^{-1} of SLO diluted 1:10 with Optimem. MTRIPs in SLO were delivered to the cells and incubated for ten minutes at 37°C . After the delivery, cells were recovered in complete DMEM media for fifteen minutes at 37°C , fixed in 4% paraformaldehyde for ten minutes at room temperature, and stained. Images were captured and probes were quantified with Volocity software. Green represents the cell structure; blue represents the nuclei (DAPI); and red represents the MTRIPs (Cy3): lower case images indicate the same cell but only with the DAPI and Cy3 channels **A**) Single cell with 2U mL^{-1} SLO MTRIP delivery with MTRIPs concentrated at 5nM **B**) Single cell with 2U mL^{-1} SLO MTRIP delivery with MTRIPs concentrated at 10nM **C**) Single cell with 2U mL^{-1} SLO MTRIP delivery with MTRIPs concentrated at 3nM **D**) Comparison of average MTRIPs in the cell for MTRIPs concentrated at 5nM, 10nM, and 30nM

The dependence of MTRIP delivery based on varying SLO concentrations were visualized in Figure 5. 1nM of RSV MTRIPs were delivered with varying concentrations of SLO at 0.2U mL⁻¹, 2U mL⁻¹, 5U mL⁻¹, 10U mL⁻¹, and 50U mL⁻¹. It was observed that minimal amounts of probe were delivered to the cells at the 0.2U mL⁻¹ SLO case. For both the 5U mL⁻¹ and 10U mL⁻¹ SLO cases, similar quantities of probe were observed in cells. However, for the 2U mL⁻¹ SLO case, a higher quantity of probe was visualized inside the cells. No notable differences in MTRIP concentration inside the cell were observed between the 2U mL⁻¹, 5U mL⁻¹, and 10U mL⁻¹ SLO cases. However, for MTRIPs delivered at 50U mL⁻¹ of SLO, an increase in intracellular MTRIP concentration was observed. From this data, it appears that the optimal MTRIP delivery concentration using minimal amounts of MTRIP and SLO can be reached using 2U mL⁻¹ of SLO. However, to effectively deliver more MTRIPs, 50U mL⁻¹ of SLO can be used on A549s with little effect on cell viability.



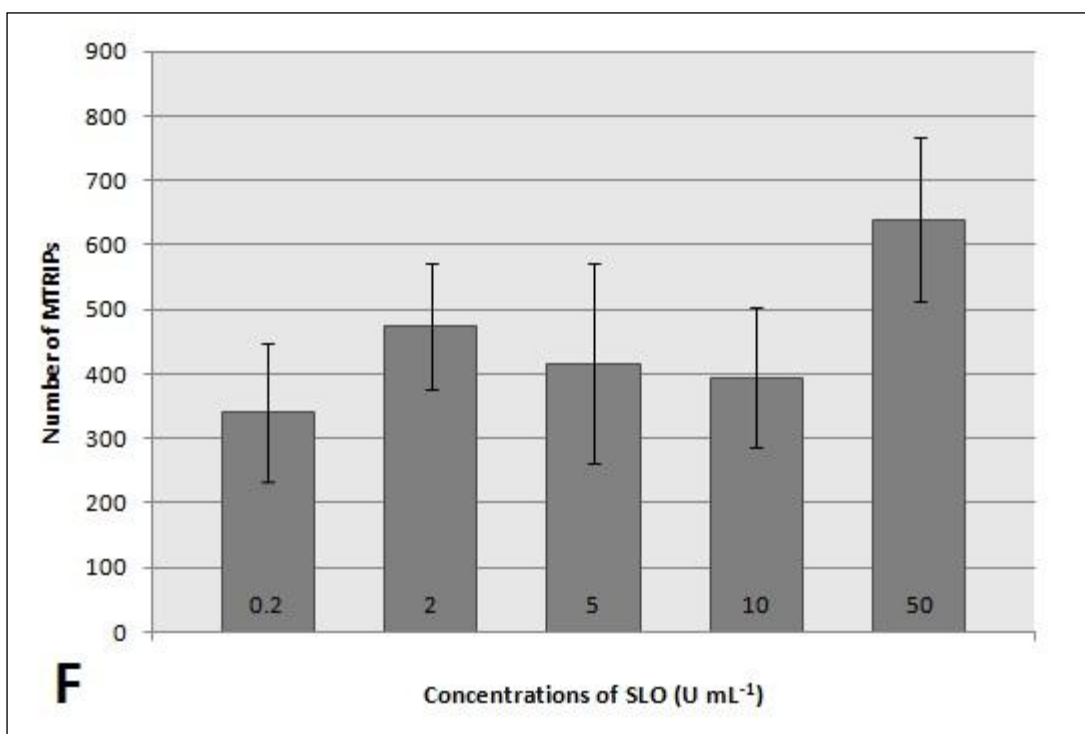
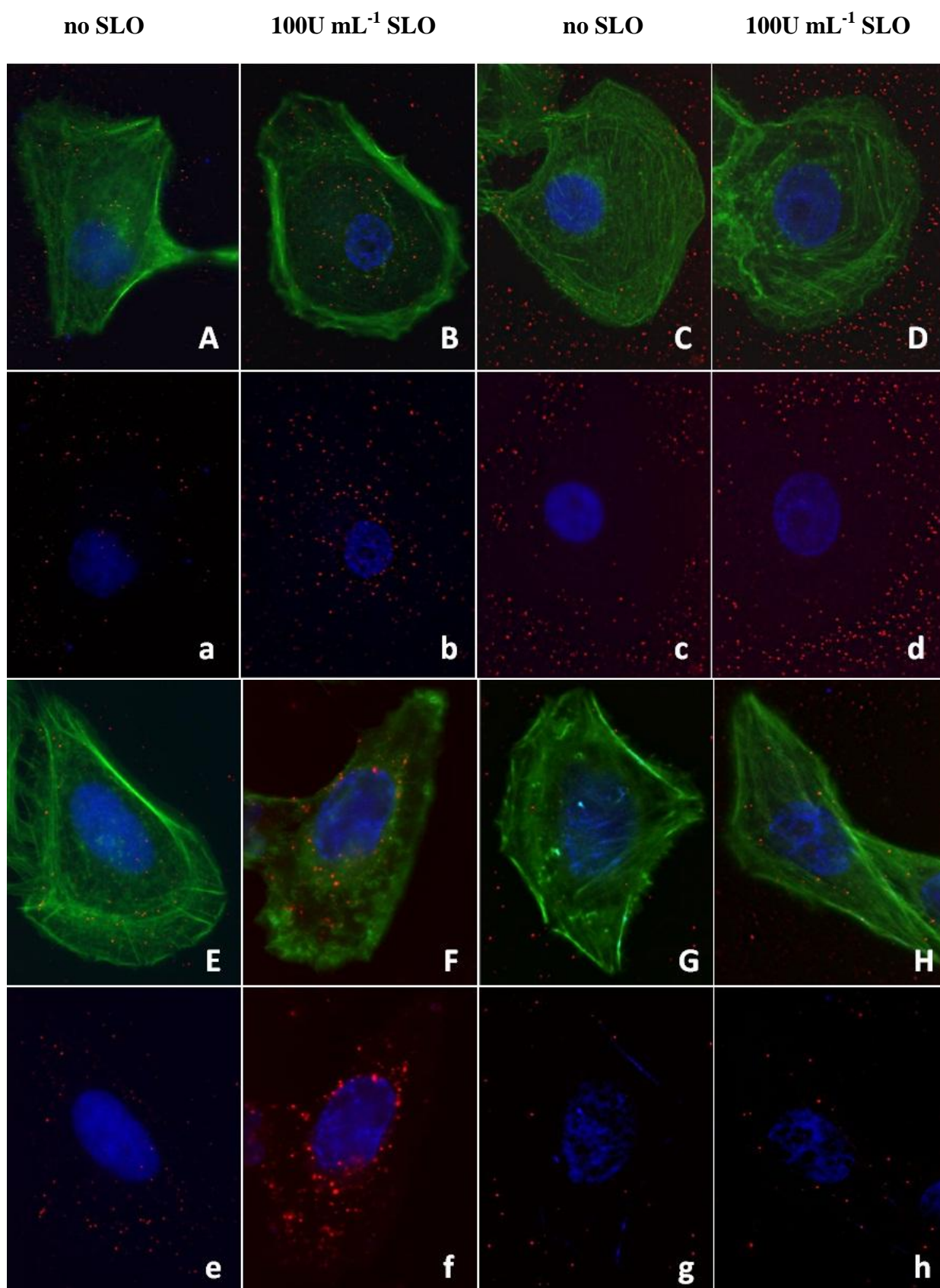


Figure 5. 1nM RSV Cy3B MTRIPs were delivered in 0.2U mL⁻¹, 2U mL⁻¹, 5 U mL⁻¹, 10 U mL⁻¹, and 50 U mL⁻¹ of SLO diluted 1:10 with Optimem in A549 cells. These cells were cultured in DMEM media with 10% FBS and with 100 U⁻¹ of penicillin and 100 mg mL⁻¹ of streptomycin. Cells were washed with Ca and Mg free PBS before MTRIP delivery with SLO. MTRIPs in SLO were delivered to the cells and incubated for ten minutes at 37°C. Cells were then recovered in complete DMEM media for fifteen minutes at 37°C, fixed in 4% paraformaldehyde for ten minutes at room temperature, and stained. Images were captured and probes were quantified with Volocity software. Green represents the cell structure; blue represents the nuclei (DAPI); and red represents the MTRIPs (Cy3): lower case images indicate the same cell but only with the DAPI and Cy3 channels **A)** Single cell with 0.2U mL⁻¹ SLO MTRIP delivery **B)** Single cell with 2U mL⁻¹ SLO MTRIP delivery **C)** Single cell with 5U mL⁻¹ SLO MTRIP delivery **D)** Single cell with 10U mL⁻¹ SLO MTRIP delivery **E)** Single cell with 30U mL⁻¹ SLO MTRIP delivery **F)** Comparison of average MTRIPs delivered at varying SLO concentrations

The effect of MTRIP delivery using SLO was also observed in varying cell types. MTRIP delivery was performed in four different cell lines: Madin-Darby Canine Kidney (MDCK) epithelial cells, African green monkey kidney (Vero) epithelial cells, human osteosarcoma cells (U2OS), and human epidermoid cancer cells (HEp2). Initially, MTRIP delivery was performed using 2U mL⁻¹ of SLO. However, no observable differences of delivery with no SLO and SLO were present. In order to examine significant changes in delivery, 100U mL⁻¹ of SLO were used to deliver 1nM of RSV MTRIPs as seen in Figure 5. A considerable difference was observed for the MDCK cell

line where a threefold increase was observed with SLO delivery than in the no SLO case. It was observed in the no SLO case of MDCKs that residual MTRIPs had a tendency to adhere to the cell surface. For Vero cells, no noticeable difference in MTRIPs was observed in the no SLO and SLO cases. Both cases displayed a similar number of MTRIPs present or adhered to the surface. Fewer MTRIPs were also observed to adhere less to the MDCK cells than the MDCK or U2OS cells. The insignificant difference between the no SLO and SLO delivery cases in the Veros may be dependent upon cell morphology. If these cells are flatter, MTRIPs may be less likely to move down the concentration gradient and passively diffuse through the created pores into the cytosolic space. In U2OS cells, a twofold increase in MTRIP concentration was observed with SLO delivery. Similar numbers of MTRIPs appeared to adhere to the surface of U2OS cells as those that adhered to the MDCK cells. In HEp2 cells, a threefold increase was observed with SLO delivery. However, the quantity of MTRIPs inside the cell was significantly less than those present in the other cell types. This characteristic may also relate to the specific cell morphology. Additionally, very few MTRIPs appeared to adhere to the cell surface. Higher concentrations of SLO seemed to work more effectively on several cell types. No significant differences in MTRIP concentrations were observed when no SLO and 2U mL^{-1} of SLO were used. However, significant differences in MTRIP concentration were observed in MDCK, U2OS, and HEp2 cells using 100U mL^{-1} of SLO.



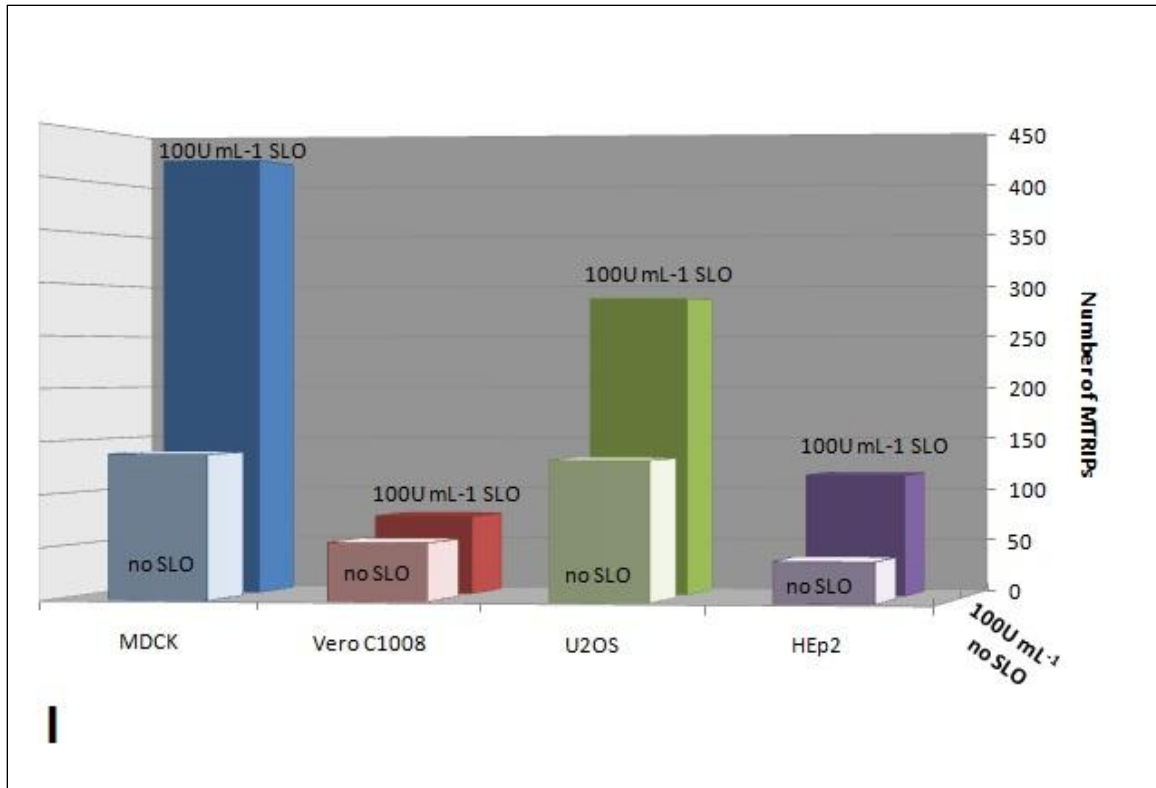


Figure 6. 1nM RSV Cy3B MTRIPs were delivered in 100U mL⁻¹ of SLO diluted 1:10 with Optimem in MDCK, Vero C1008, U2OS, and HEp2 cells. These cells were cultured in DMEM media with 10% FBS and with 100 U⁻¹ of penicillin and 100 mg ml⁻¹ of streptomycin. Cells were washed with Ca and Mg free PBS before MTRIP delivery with SLO. MTRIPs in SLO were delivered to the cells and incubated for ten minutes at 37°C. Cells were then recovered in complete DMEM media for fifteen minutes at 37°C, fixed in 4% paraformaldehyde for ten minutes at room temperature, and stained. Images were captured and probes were quantified with Volocity software. Green represents the cell structure; blue represents the nuclei (DAPI); and red represents the MTRIPs (Cy3): lower case images indicate the same cell but only with the DAPI and Cy3 channels **A)** Single MDCK cell with no SLO MTRIP delivery **B)** Single MDCK cell with 100U mL⁻¹ SLO MTRIP delivery **C)** Single Vero cell with no SLO MTRIP delivery **D)** Single Vero cell with 100U mL⁻¹ SLO MTRIP delivery **E)** Single U2OS cell with no SLO MTRIP delivery **F)** Single U2OS cell with 100U mL⁻¹ SLO MTRIP delivery **G)** Single HEp2 cell with no SLO MTRIP delivery **H)** Single HEp2 with 100U mL⁻¹ SLO MTRIP delivery **I)** Comparison of average MTRIPs delivered in varying cell types

CONCLUSIONS

Differences in MTRIP delivery were observed in A549 cells by manipulating the MTRIP delivery concentration and partially adjusting the SLO delivery solution. More MTRIPs were observed in the cytoplasm of cells when higher concentrations of MTRIPs were available, as in the 30nM delivery case in Figure 4C. Additionally, the abundance of MTRIPs in the SLO delivery environment positively correlated to the quantity of MTRIPs observed in the cytoplasm. Altering the concentration of MTRIPs in the SLO delivery solution was effective in increasing the cytosolic concentration of MTRIPs in cells; however, adjusting SLO concentrations did not dramatically increase the cytosolic content of MTRIPs in cells. No distinguishable differences in MTRIP concentration were observed in the 2, 5, and 10U mL⁻¹ SLO concentrations. There was no positive correlation between increased SLO concentration and cytosolic MTRIP concentrations. Of the lower SLO concentrations, 2U mL⁻¹ of SLO had more effective delivery than that of the 0.2, 5, and 10U mL⁻¹ of SLO cases. The 50U mL⁻¹ SLO delivery case did display a significant increase in intracellular MTRIP concentration. This may indicate that only much higher concentrations of SLO are effective in binding to pores and creating the maximum amount passages for MTRIPs to travel through.

Additionally, varying cell types responded differently to the effects of SLO delivery. MDCK, Vero, U2OS, and HEp2 cells presented no significant changes with untargeted MTRIPs delivered at 2U mL⁻¹ of SLO. In order to effectively determine changes in MTRIP concentration, 100U mL⁻¹ of SLO was used and resulted in different results for each cell type. MDCK, U2OS, and HEp2 cells were shown to have a twofold or threefold increase of MTRIPs in the cytoplasm. Vero cells did not display any

significant difference in MTRIP delivery with no SLO and with SLO. Adherence of MTRIPs to the cell membrane also varied with cell types. More MTRIPs were observed on the surface of the MDCK and U2OS cells than on Vero or HEp2 cells. It is currently not known why these fluorescent probes adhere to only specific cell surfaces, but it may occur due to varying quantities of different membrane proteins available on differing cell types. Future studies to determine why cell type delivery differs could include quantifying the amount of available cholesterol for SLO to bind to in differing cell types or determining what membrane components affect the binding of MTRIPs to the cell surface for differing cell types. More information on these factors could promote more efficient probe delivery in more cell types.

Observations of SLO delivery dynamics with MTRIPs provides detailed information on how to effectively deliver fluorescent probes to an intracellular environment. Optimizing the amount of probes that can be delivered is crucial to targeting all of the available endogenous RNA or proteins within the cell. Efficiently targeting all the available mRNA or proteins will allow for a more accurate representation of molecular which in turn can lead to better, long-term imaging to visualize real-time molecular processes and can eventually alleviate the gap of understanding in molecular mechanisms.

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